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# Effects of Cholesterol Concentrations on Egg-phosphatidylcholine – Dihexadecyl-phosphate Liposomes Studied by CW ESR and FT – ESEEM Spectroscopy

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The influence of cholesterol on water penetration depth as well as on the motional properties of spin-labelled stearic acids (*n*-SASL, *n* = 5, 7, 10, 12, 16) in liposomes composed of egg-yolk phosphatidylcholine (egg-PC) and dihexadecyl-phosphate (DHP) was studied by electron spin echo envelope modulation (ESEEM) and continuous wave electron spin resonance (CW ESR) spectroscopy. An increased amount of $D_2O$ molecules in the neighbourhood of the nitroxide moiety bound at 5- and 7-SASL, concomitant with an absence of $D_2O$ at 10-SASL and 16-SASL was determined in the presence of cholesterol in $x$ = 8, 15, 29, 38, and 45 % (mole fraction). At 16-SASL, presence of cholesterol at any of the concentrations examined caused increased amplitude of the librational motion of the nitroxide moiety as compared to that amplitude in the absence of cholesterol. These results indicate that the inclusion of cholesterol increases water penetration in the polar region of the bilayer and influences structural change in the hydrophobic core of membrane.

*Keywords*
• egg-phosphatidylcholine
• cholesterol
• spin label
• CW ESR
• electron spin echo envelope modulation (ESEEM) spectroscopy

## INTRODUCTION

The physical properties of model phospholipid-cholesterol membranes were extensively studied by different methods on two- and three- component systems composed of pure phospholipids with saturated and unsaturated fatty acid chains. These studies have been described in several review articles appearing recently in the literature.[1–6] Molecular organization of phospholipids was found to be strongly dependent on cholesterol concentration. It is accepted that cholesterol induces liquid–ordered phases that can coexist with liquid–disordered fluid phases.[7,8] Ordered and disordered phases have also been confirmed to coexist in plasma membrane vesicles of RBL-2H3 mast cells.[9] Chiu *et al.*[10] studied the influence of cholesterol concentrations from $x$ = 4.0 % to $x$ = 50.0 % (mole fraction) on the molecular area of dipalmitoyl-phosphatidylcholine (DPPC). Even at $x$ = 4 % of cholesterol, the formation of subunits containing cholesterol hydrogen-bonded to the DPPC molecule was suggested, as a composite particle that aggregates with other such composites. It was also suggested that one cholesterol molecule can condense about three POPC molecules at 2 °C.[11] The effects of cholesterol on the dynamics of cholestan spin probe in various DMPC/POPC/cholesterol ternary mixtures were studied by Freed and co-workers.[12] Lateral diffusion of cholesterol analogue spin probe was found to decrease by a factor of four even at $x$ = 10 % of cholesterol as compared to that of $x$ = 0 %. So even at low concentrations, cholesterol influences the phospholipids' molecu-

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lar organisations in vesicles of pure phospholipids.[13] On the other hand, when cholesterol is added to lipid mixtures, such as egg-PC containing different fatty acid chains, a simple prediction of the effects of inclusion of cholesterol into the lipid bilayer of liposomes is not clear.

It is not yet fully understood how cholesterol affects properties of water interface in membranes, though water-phospholipid interactions have been extensively studied[14,15] and recently reviewed.[16,17] A method based on electron spin echo envelope modulation (ESEEM) was improved by the technique of Fourier transformation (FT-ESEEM).[18–21] The essence of the method is a hyperfine coupling of the unpaired electron of the nitroxide moiety with surrounding $D_2O$ molecules. A sigmoidal dependence of the water penetration depth into the liposome on the position of the doxyl moiety in the fatty acid chain was reported.[20,21] By three-pulse stimulated echo in liposomes composed of dipalmitoylphosphatidylcholine, the mid-point of the sigmoidal profile was found to be shifted towards the membrane centre in membranes without cholesterol as compared to those with $x$ = 40 % of cholesterol.[22]

Multi-lamellar liposomes composed of egg-PC – DHP (in mole ratio $r$ = 7 : 1) were used to protect liposome membrane in the presence of glycine from lysis by chlorpromazine,[23] and recently to study interactions with adamantyl-tripeptides, peptidoglycan monomer[24] and ovalbumin.[25] Results[19,26] on the water penetration depth into multi-lamellar liposomes containing egg-PC and DHP obtained by FT-ESEEM pointed to the importance of cholesterol for the formation of an efficient hydrophobic barrier in liposomes containing a mixture of fatty acid chains of phospholipids such as egg-PC. DHP has two saturated chains and a negative charge on the phosphate oxygen. The negative charge and saturated fatty acids of DHP may influence domains formation with cholesterol as well as the surface properties of liposomes.

This work examines the effects of different cholesterol concentrations on physical properties of multi-lamellar liposomes (composed of egg-PC and DHP), such as water penetration depth into the interfacial region of the lipid bilayer. Also, possible structural changes in the hydrophobic core due to the presence of cholesterol are examined using spin labels, *n*-doxyl stearic acids ($n$ = 5, 7, 10, 12, 16). FT-ESEEM experiments were undergone at 35 K in order to freeze librational motion of the hydrogens in the doxyl group of the spin labels. CW ESR experiments were performed in the temperature range from 105 to 293 K.

## EXPERIMENTAL

### *Materials*

Egg-yolk L-α-Phosphatidylcholine (egg-PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Egg-PC contains a mixture of acyl chains of length ranging from 16 to 22 carbon atoms with varying number of double bonds, but about 98 % phospholipids with one saturated and one unsaturated chain. Cholesterol (5-cholesten-3$\beta$-ol), dihexadecyl-phosphate (DHP) and all *n*-doxyl stearic acids ($n$ = 5, 7, 10, 12, 16) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Chemicals for the phosphate buffer (0.1 mol dm$^{-3}$, $NaH_2PO_4$ + $Na_2HPO_4$, pH = 7.1) and PBS (0.15 mol dm$^{-3}$, $NaH_2PO_4$ + $Na_2HPO_4$ + 0.15 mol dm$^{-3}$ NaCl, pH = 7.4) were of analytical grade, from Kemika (Zagreb, Croatia). The $D_2O$ for FT-ESEEM experiments was from Aldrich Chemical Company (Milwaukee, USA).

### *Preparation of Multi-lamellar Liposomes*

Egg-PC, DHP and *n*-doxyl stearic acids (*n*-SASL) were dissolved in chloroform / methanol (volume ratio $\psi$ = 1 : 1). The mole ratio of egg-PC to DHP was always 7 : 1, the mole fraction of *n*-SASL was 0.2 %, and the mole fractions of cholesterol were $x$ = 0, 8, 15, 29, 38 and 45 %. Lipid films were formed by removal of the solvent using nitrogen gas flow. To ensure complete evaporation of the solvent, samples were evacuated by mechanical pump overnight. After hydration in either PBS or in 0.1 mol dm$^{-3}$ phosphate buffer (both were prepared in $D_2O$ for ESEEM experiments), lipid suspensions were stirred and left overnight at 4 °C to swell and stabilize. In order to remove oxygen present in the samples, nitrogen gas was blown through them for 10 minutes. The samples were rapidly frozen, using liquid helium for ESEEM experiments and liquid nitrogen for low temperature CW ESR experiments.

### *CW ESR and ESEEM Experiments*

CW ESR measurements were performed on a Varian E-9, X-band (9.5 GHz) spectrometer (microwave power 10 mW, modulation amplitude 0.13 mT), equipped with a Bruker variable temperature control unit. The spectra were recorded with digital acquisition, ESR-Ware data.[27] Sample capillaries were inserted into the standard 4 mm diameter quartz tubes and centred in a TE102 ESR cavity.

Dependence of the anisotropic components of the hyperfine splitting tensor on the solvent polarity as well as on the librational motion of N-O bond of the nitroxide moiety is described by relation:[28–30]

$$A'_{zz} = A_{zz} - (A_{zz} - A_{xx}) \langle\alpha^2\rangle \quad (1)$$

$A'_{zz}$ is a motionally-averaged hyperfine tensor element derived from the CW ESR spectra. In order to determine $\langle\alpha^2\rangle$, $A_{zz}$ (the rigid limit hyperfine splitting) is derived by linear extrapolation of the data to zero temperature by measuring the temperature dependence of $A'_{zz}$. $A_{xx}$ is taken from the crystal structure.[30] $\langle\alpha^2\rangle$ varied within ± 0.003 rad$^2$.

FT-ESEEM experiments (two-pulse Hahn-echo decay $\pi/2 - \tau - \pi - \tau$ – echo; $\pi/2$ = 12 ns, $\pi$ = 24 ns, $\tau$ = 120 ns in 4 ns steps) were carried out with a Bruker ELEXSYS E580 Fourier Transform X-band ESR spectrometer equipped with a standard Oxford Instruments model DTC2 tempera-

ture controller, at 35 K. The phase-memory time, $T_{2m}$, was determined by fitting the maximum echo amplitude as a function of $2\tau$ with a simple exponential decay.

The use of Fourier transform electron spin echo envelope modulation (FT-ESEEM) for measurements of the water ($D_2O$) penetration depth into lipid bilayer is based on the deuteron effect on the $^{14}N$-hyperfine splitting constant of the spin label. Anisotropic hyperfine interactions between N-O and $D_2O$ modulate the electron spin echo decay, and the deuteron spin transitions are observed at deuteron Larmor frequencies $\nu_L(D)$ = 2.288 MHz and $2\nu_L(D)$. The depth of this modulation depends on the distance of $D_2O$ from the N-O bond as well as on the number of the nearby deuterons. In the frequency domain, the ratio of amplitudes of the lines at Larmor frequencies of deuterons (*D*) to protons (*H*), *D*/*H*, gives information about the number of water molecules bound to the N-O moiety.[17–19] Concentrations of the spin labels used in the experiments were kept low enough (approx. $x$ = 0.2 %) to avoid electron spin-spin interactions,[25] thus it may be assumed that the loss of phase coherence was only *via* dipolar interactions between magnetic moments of the electron spin and H or D nuclei. $T_{2m}$ was determined by fitting a single exponential to echo decay, as described previously.[18] $T_{2m}$ varied within ± 50 ns.

## RESULTS AND DISCUSSION

In order to determine water penetration depth into liposomes as a function of cholesterol concentration, FT-ESEEM was used. The ESEEM spectrum of 5-doxyl stearic acid (5-SASL) in liposomes containing no cholesterol is displayed in the time domain in Figure 1A, and in the frequency domain (FT-ESEEM) in Figure 1B. The spectra were taken at 35 K in order to freeze all the dynamics that may influence $T_{2m}$, *e.g.* librational motion of the hydrogens of the doxyl group. The ratio of the intensities of deuterons to protons (*D*/*H*) in liposomes as a function of cholesterol concentration at $\nu_L(D)$ = 2.288 MHz and $\nu_L(H)$ = 14.902 MHz (Figure 1B) in the spectra of *n*-SASL ($n$ = 5, 7, 10, 16) is shown in Figure 2. In the absence of cholesterol, liposomes spin-labelled with 5-, 7- and 10-SASL revealed similar *D*/*H* values (0.5) while no deuteron lines were detected with 16-SASL. The addition of $x$ = 8 and 15 % of cholesterol caused an increase of *D*/*H* to 0.8 and 0.9 respectively for 5-SASL, while for 10-SASL caused a decrease of *D*/*H* from 0.5 to 0.35 at $x$ = 8 % and from 0.5 to 0.2 at $x$ = 15 %. At $x$ = 29 % of cholesterol and above, *D*/*H* values of about 2.0 were determined for 5-SASL, while no deuteron frequency was detected for 10-SASL and 16-SASL. This suggests an increased amount of $D_2O$ in the near-neighbourhood of the nitroxide moiety in 5- and 7-SASL, and an absence of $D_2O$ at 10-SASL.

$D_2O$ bound to nitroxide moiety also influences phase memory time, $T_{2m}$, as shown in Table I. For 5-SASL, $T_{2m}$ increased from about 700 ns in the absence of cholesterol to 1100 ns in the presence of $x$ = 38 %. This supports the increased binding of $D_2O$ to the nitroxide moiety. For 16-SASL, no deuteron lines were observed in the frequency domain at any of the cholesterol concentrations and $T_{2m}$ remained at about 220 ns in the whole range of cholesterol concentrations examined, which means that, at this position no $D_2O$ was detected (Table I). Although *D*/*H* ratio suggests an increased $D_2O$ bound to the N-O bond, $T_{2m}$ for 7-SASL deviates from the predicted values obtained for 5-SASL. This may be due to different

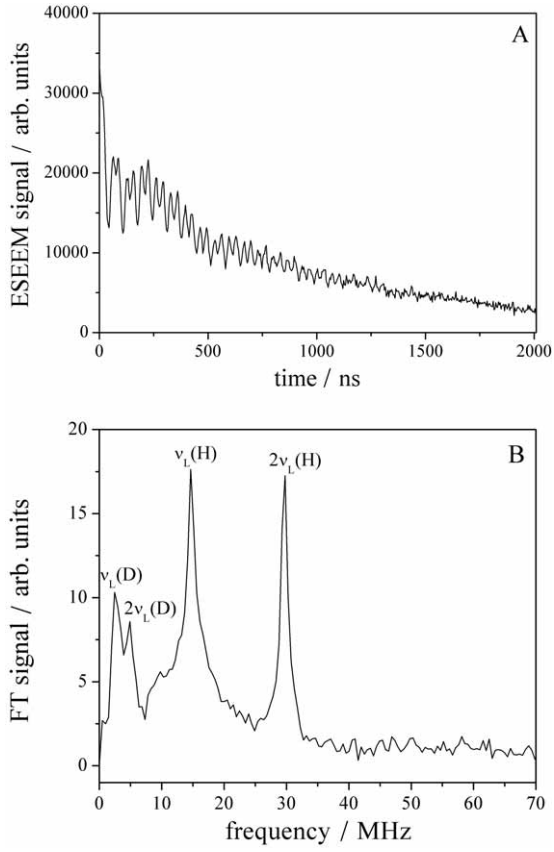


Figure 1. A – Time domain ESEEM, and B – FT-ESEEM spectra of 5-SASL incorporated in multi-lamellar liposomes (egg-PC : DHP = 7 : 1) in 0.1 mol dm$^{-3}$ phosphate buffer ($D_2O$, pH = 7.1). Spectra were taken at 35 K.

TABLE I. Phase memory time ($T_{2m}$) as a function of cholesterol concentration

| Mole fraction of cholesterol | $T_{2m}$ / ns | | | |
|---|---|---|---|---|
| | 5-SASL | 7-SASL | 10-SASL | 16-SASL |
| 0 | 720 | 830 | 570 | 230 |
| 8 | 700 | 930 | 420 | 210 |
| 15 | 760 | 700 | 390 | 210 |
| 29 | 930 | 680 | 350 | 220 |
| 38 | 1120 | 930 | 370 | 250 |
| 45 | 1020 | 720 | 400 | 250 |

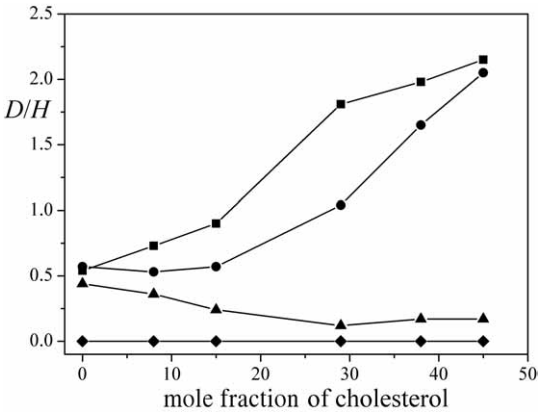


Figure 2. – (*D/H*) ratio as a function of mole fraction of cholesterol for *n*-SASL (*n* = 5 (squares), 7 (circles), 10 (triangles), 16 (diamonds)), in multi-lamellar liposomes (egg-PC : DHP = 7 : 1), 0.1 mol dm$^{-3}$ phosphate buffer ($D_2O$, pH = 7.1). Within different liposome preparations of the same composition *D/H* were determined with the error $\Delta D/H = \pm 0.1$.

positions of this spin label in the bilayer in the presence of different cholesterol concentrations.

Hydrophobic barrier (opposite of polarity profile) has usually been studied by CW ESR in the frozen state by measuring the hyperfine splitting constant, $2A'_{zz}$ (Figure 3). In accordance with previously reported results[28] the spectra were taken at 105 K. The ESR spectra of 16-SASL in multi-lamellar liposomes (A in the absence of cholesterol, B with $x$ = 8 %) are shown in Figure 3. A decrease in $2A'_{zz}$ from 6.57 mT to 6.32 mT in the presence of $x$ = 8 % of cholesterol suggests an influence of cholesterol on the hydrophobic core of the liposome. $2A'_{zz}$, as determined from the spectra of *n*-SASL in liposomes containing $x$ = 0 % (circles) and 38 % (squares) of cholesterol, is shown in Figure 4 (spectra were taken at 105 K). A sigmoidal dependence of $2A'_{zz}$ on the position of the doxyl moiety in the fatty acid chain was obtained. Higher $2A'_{zz}$ values for 5- and 7-SASL in the presence $x$ = 38 % of cholesterol are due to a greater number of water molecules bound at the N-O moiety as compared to liposomes in the absence of cholesterol. This is in accordance with FT-ESEEM results presented in Figure 2.

On the other hand, higher $2A'_{zz}$ values for 12- and 16-SASL in the absence of cholesterol (circles) than in its presence (Figure 4) cannot be due to the water present in the neighbourhood of the N-O moiety, as no water was determined at these positions by FT-ESEEM. Differences in motion of the nitroxide moiety in the lipid core may be proposed.

The parameter $A'_{zz}$ was used to measure the amplitude of librational motion, $\langle\alpha^2\rangle$, of the N-O moiety of 14-PCSL spin label in DPPC in the presence of $x$ = 50 % cholesterol, and $\langle\alpha^2\rangle$ of 0.015 rad$^2$ was obtained at 100 K.[29] For 16-SASL in egg-PC-DHP liposome at 105 K, $\langle\alpha^2\rangle$ of 0.006 rad$^2$ was calculated in the absence of cholesterol, and 0.022 rad$^2$ at $x$ = 38 %, from relation (1).

$2A'_{zz}$ measured from the spectra of 5- and 16-SASL in liposomes as a function of different cholesterol concentrations are compared in Figure 5. The spectra were recorded at 188 K. In the case of 5-SASL (Figure 5, squares), $2A'_{zz}$ values increased from 6.7 mT at $x$ = 15 % to 6.95 mT at $x$ = 38 % cholesterol. This is in accordance with an increased amount of water in the near-neighbourhood of the nitroxide moiety determined by FT-ESEEM. In the case of 16-SASL, a decreased $2A'_{zz}$ value, from 6.57 mT to 6.32 mT, obtained at all cholesterol concentrations ($x$ = 8, 15, 29, 38, 45 %) (Figure 5, circles) may have been due to increased librational motion of the nitroxide moiety. At 188 K in the absence of cholesterol, $\langle\alpha^2\rangle$ of 0.0056 rad$^2$ was determined, *i.e.* a

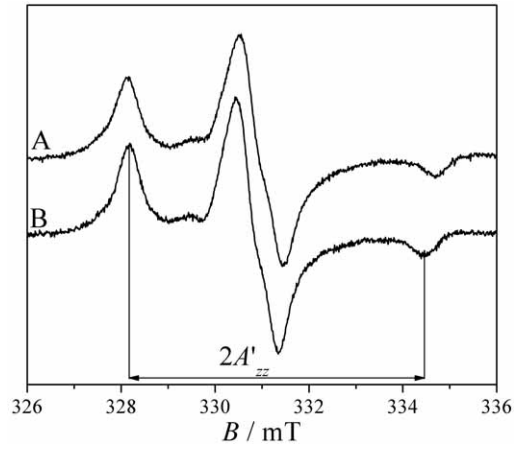


Figure 3. – ESR spectra of 16-SASL in multi-lamellar liposomes (egg-PC : DHP = 7 : 1) in PBS buffer, pH = 7.4, with (A) $x$ = 0 % of cholesterol and (B) $x$ = 8 % of cholesterol. Spectra were taken at 188 K. $2A'_{zz}$ parameter is indicated by arrows.

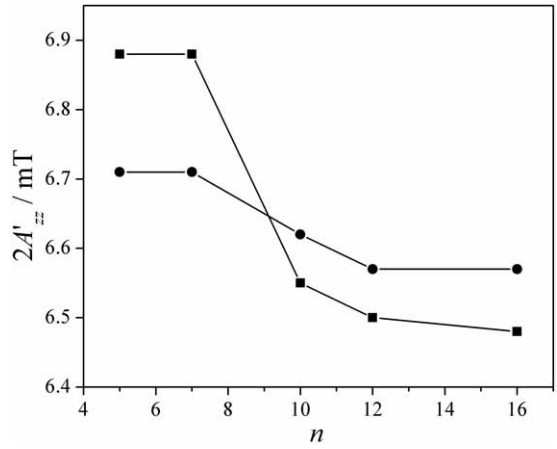


Figure 4. – $2A'_{zz}$ parameter as a function of position (*n*) of the nitroxide moiety on the stearic acid spin label (*n*-SASL) incorporated into multi-lamellar liposomes (egg-PC : DHP = 7 : 1) with $x$ = 0 % (circles) and $x$ = 38 % of cholesterol (squares) in PBS buffer, pH = 7.4. The spectra were taken at 105 K.

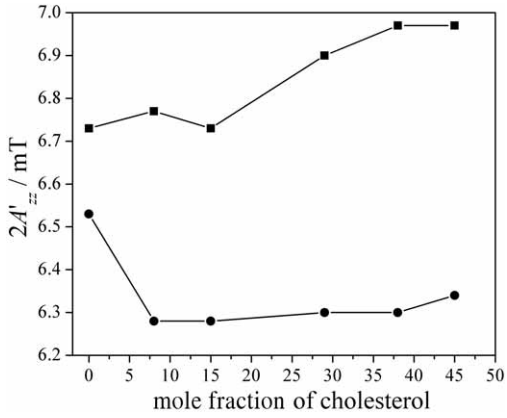


Figure 5. – Dependence of $2A'_{zz}$ parameter of 5-SASL (squares) and 16-SASL (circles) on cholesterol concentrations in multi-lamellar liposomes (egg-PC : DHP = 7 : 1) in PBS buffer, pH = 7.4. Spectra were taken at 188 K.

similar value as at 105 K, while at all the other cholesterol concentrations $<\alpha^2>$ increased to 0.045 rad$^2$. In the hydrophobic core of the liposomes, at 16-SASL, the presence of cholesterol at any of the concentrations examined caused a similar amplitude of librational motion of the nitroxide moiety. A lesser amplitude of librational motion by an order of magnitude was determined in the absence of cholesterol.

Temperature dependence of the parameter $2A'_{zz}$ in the spectra of 5-SASL, reflecting water penetration depth into the interfacial region at low temperatures (Figure 6A), and of 16-SASL, reflecting librational motion of the nitroxide moiety in the hydrophobic core (Figure 6B), are compared for different cholesterol concentrations. For 5-SASL, decrease in the $2A'_{zz}$ parameter started at higher temperatures in liposomes with $x$ = 0, 8, 15 % of cholesterol than in liposomes with $x$ = 29, 38, 45 % of cholesterol. A similar temperature dependence was obtained for $2A'_{zz}$ in 5-SASL (Figure 6A) with $x$ = 0, 8, 15 % of cholesterol, with constant $2A'_{zz}$ = 6.6 mT between 190 and 250 K (close to the gel-to-liquid crystal phase transition temperature of pure egg-PC). For $x$ = 29, 38 and 45 % cholesterol concentrations, $2A'_{zz}$ = 6.9 mT remained unchanged up to about 240 K.

In the case of 16-SASL, a different temperature dependence was obtained for $2A'_{zz}$ with $x$ = 0 % as compared to all the other cholesterol concentrations, *i.e.* at $x$ = 0 %, $2A'_{zz}$ = 6.55 mT while $2A'_{zz}$ = 6.30 mT at $x$ = 8, 15, 29, 38, 45 % in the temperature interval 190 – 220 K. Sigmoidal decrease of $2A'_{zz}$ was observed between 230 and 293 K. Above 270 K, $2A'_{zz}$ = 3.25 mT for $x$ = 0, 8, 15 % of cholesterol and $2A'_{zz}$ = 3.75 mT for $x$ = 29, 38 and 45 %. The temperature dependence of both spin labels suggests the formation of two motionally different phases above $x$ = 29 % of cholesterol.

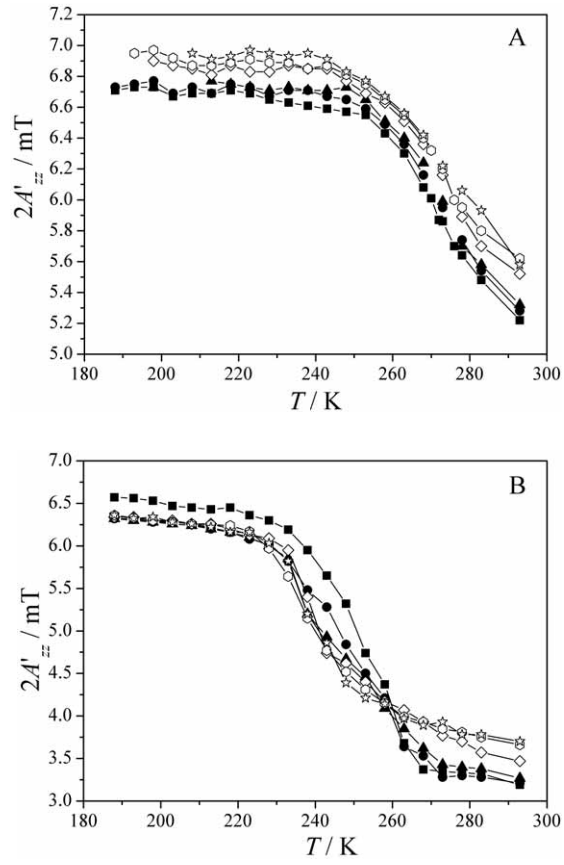


Figure 6. – Temperature dependence of $2A'_{zz}$ from CW ESR spectra of: A – 5-SASL; B – 16-SASL, incorporated into multi-lamellar liposomes (egg-PC : DHP = 7 : 1) in PBS buffer, pH = 7.4, as a function of different cholesterol concentrations, $x$ = 0 % – squares, 8 % – circles; 15 % – triangles; 29 % – diamonds; 38 % – hexagons; 45 % stars.

## CONCLUSIONS

An increased number of $D_2O$ molecules in the near neighbourhood of N-O at 5- and 7-SASL concomitant with the absence of $D_2O$ at 10-SASL and at 16-SASL was determined in the presence of cholesterol from $x$ = 8 to 45 % by FT-ESEEM. In accordance with the results obtained by FT-ESEEM, sigmoidal dependence of the polarity profile was also determined by CW ESR.

In the hydrophobic core of the liposome at 16-SASL, presence of cholesterol caused an increased amplitude of librational motion ($<\alpha^2>$) of the nitroxide moiety from 0.0056 rad$^2$ in the absence of cholesterol to 0.045 rad$^2$ at all cholesterol concentrations examined. These results suggest that changes in the polar surface induced by inclusion of cholesterol are also reflected in the hydrophobic core of the lipid bilayer.

Phase memory time, $T_{2m}$, as well as *D/H* ratio, are suggested as a parameter for determining water penetration depth into phospholipid bilayer.

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## SAŽETAK

### Proučavanje utjecaja koncentracija kolesterola na liposome jajčanog fosfatidilkolina i dicetil-fosfata pomoću CW ESR i FT – ESEEM spektroskopija

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Spektroskopskim je metodama, ESEEM i CW ESR, proučavan utjecaj prisutnosti kolesterola na dubinu prodiranja vode u liposome sastavljene od jajčanog fosfatidilkolina i diheksadecil-fosfata pomoću spinski označenih stearinskih kiselina (*n*-SASL, *n* = 5, 7, 10, 12, 16) ugrađenih u liposome. Ugrađivanjem kolesterola u molnim udjelima *x* = 8, 15, 29, 38, 45 % u liposome opaženo je povećanje broja $D_2O$ molekula u bliskoj okolini N-O grupe nitroksilnog radikala u 5- i 7-SASL-u, i odsustvo $D_2O$ molekula u okolini N-O grupe 10-SASL-a i 16-SASL-a. Prisutnost bilo koje koncentracije kolesterola u liposomu uzrokuje povećanje amplitude libracijskog gibanja N-O grupe u 16-SASL-u. Rezultati sugeriraju, da ugrađivanje kolesterola u liposome ne samo da uzrokuje povećanje prodiranja vode u polarni dio dvosloja membrane nego uzrokuje i strukturnu promjenu hidrofobne jezgre.